# Carotenoid profiling of *Hordeum chilense* grains: the parental proof for the origin of the high carotenoid content and esterification pattern of tritordeum.

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**Chemical compounds studied in this article:**

β-Carotene (PubChem CID: 5280489); Zeaxanthin (PubChem CID: 5280899); Lutein (PubChem CID: 5368396)

**Abstract**

The outstanding high carotenoid content of the tritordeum (×*Tritordeum* Ascherson et Graebner) grains, a promising novel cereal derived from the crossing of durum wheat and the wild barley *Hordeum chilense*, has previously been assigned as a character derived from the genetic background of its wild parent. The carotenoid profile of *H. chilense*, especially the lutein esters, presented in this study provide biochemical evidences to confirm this affirmation, being the first time that the individually carotenoid profile of this cereal has been characterized. The total carotenoid content (6.14 ± 0.12 μg/g) and the individual carotenoid composition were very similar to the tritordeum grains, with lutein being the major carotenoid (88%; 5.38 ± 0.11 μg/g) and very low levels of β-carotene. In contrast to tritordeum, *H. chilense* presented a considerable amount of zeaxanthin (12%; 0.74 ± 0.01 μg/g). Up to 55% of lutein was esterified with palmitic (C16:0) and linoleic (C18:2) acids, presenting a characteristic acylation pattern, in agreement with the tritordeum one, and composed by four monoesters (lutein 3´-*O*-linoleate, lutein 3-*O*-linoleate, lutein 3´-*O*-palmitate and lutein 3-*O*-palmitate) and four diesters (lutein dilinoleate, lutein 3´-O-linoleate-3-*O*-palmitate, lutein 3´-*O*-palmitate-3-*O*-linoleate, lutein dipalmitate). These data may be useful in the field of carotenoid biofortification of cereals.

## 1. Introduction

Carotenoids pigments are lipophilic molecules responsible for the red, orange and yellow colors of most fruits and vegetables and certain animals. The latter, including the humans, are unable to synthesize carotenoids *de novo*, so they need to incorporate them in the diet. Carotenoids play their basic functions as light collectors in the photosynthetic apparatus of plants, besides preventing oxidative damage as potent antioxidants. As a result of the antioxidant property, important functions for human health are derived, such as the prevention of certain degenerative and chronic diseases (Fernández-García et al., 2012). Although the cereal grains have a relatively low carotenoid content compared to most fruits and vegetables, the daily intake of cereals and cereal derived products by the majority of the population, makes these staple foods as a non-negligible and affordable source for carotenoids and other phytochemicals (Graham and Rosser, 2000), becoming ideal vehicles to be used in biofortification and nutritional strategies (Bai et al., 2011).

Since the beginning of twentieth century, an increasing interest has been raised among cereal breeders for the development of interspecific hybrids in order to obtain new cereals with increased phytochemical contents and improved agronomic performance and technological qualities. One of the success stories in cereal breeding is the generation of tritordeum (×*Tritordeum* Ascherson et Graebner), the fertile amphiploids derived from the crossing of durum wheat and a wild barley (*Hordeum chilense* Roem. & Schult.) (Martín and Sánchez-Monge Laguna, 1982; Martín et al., 1999). *H. chilense* is a wild diploid barley (2n = 2x = 14) belonging to the section *Anisolepsis Nevski*, being an extremely variable species included in a heterogeneous group of South American species of the genus *Hordeum*, carrying the H genome. The use of this wild cereal in breeding programs has focused on two main areas; first, the

development of *Tritordeum* amphiploids between *H. chilense* and tetraploid (*T. turgidum* Desf.) or hexaploid (*T. aestivum* L.) wheats with the aim of obtaining a new functional cereal; and second, the introgression to wheat of new traits of interest (agronomical, technological, nutritional, etc). One of the main interests of this species is its potential for increasing the carotenoid content in durum wheat (Rodríguez-Suarez et al., 2011). The color of durum wheat semolina is the mainly due to the carotenoid pigments of the grains, being considered an important quality criterion with regard to pasta production (Hentschel et al., 2002). This quality trait has been frequently assessed as YPC (yellow pigment content). The genetic variation of endosperm color trait in tritordeum and its relationship to the level of pigments in both parental species, *H. chilense* and durum wheat has also been characterized (Álvarez et al., 1999).

The small grains of *H. chilense* are characterized by a high level of carotenoids, presenting at least two loci (QTLs) for the pigment content trait located on the $2H^{ch}$ and $7H^{ch}$ chromosomes, existing a high genetic variability for this trait (Álvarez et al., 1998; Álvarez et al., 1999; Atienza et al., 2004). More recently, twelve genes related to endosperm carotenoid content in grasses (*Dxr, Hdr, Ggpps1, Psy2, Psy3, Pds, Zds, e-Lcy, b*-Lcy, *Hyd3, Ccd1 and Ppo1*) have been mapped in *H. chilense*, and additionally a new main region associated with YPC has been identified in $3H^{ch}$ chromosome (Rodríguez-Suarez and Atienza, 2012). These finding provide the first steps towards the implementation of a MAS (Marker Assisted Selection) program for identifying genes determining a higher carotenoid in *Tritordeum*, and for developing of new tools and strategies for transferring these genes and traits from selected amphiploids to wheat lines under breeding programs (Atienza et al., 2007a). In previous works, it has been demonstrated that the advanced tritordeum lines showed carotenoid levels up to 8-times higher than their parental durum wheat cultivars, with lutein being the major pigment

(Atienza et al., 2007b; Mellado-Ortega and Hornero-Méndez, 2012). Moreover, a high proportion of lutein (up to 40%) in tritordeum is presented as mono- and diesters (homo- and heterodiesters) with a specific set of two fatty acids, palmitic and linoleic acids, for which different regioisomers of monoesters and heterodiesters have been identified and characterized in tritordeum for the first time in a cereal grain (Mellado-Ortega and Hornero-Méndez, 2012). There are experimental evidences suggesting that the esterification of xanthophylls is an important mechanism and strategy in vegetables for sequestering and accumulating these lipophilic compounds within the plastids (Fernandez-Orozco et al., 2013; Hornero-Méndez and Mínguez-Mosquera, 2000). According to previous reports, the esterification of xanthophylls, such as lutein and β-cryptoxanthin, with fatty acid increased their stability against heat and light (Fu et al., 2010; Subagio et al., 1999), and preserved the antioxidant activity similar to the free carotenoid (Subagio and Morita, 2001). Therefore the correct understanding of the biochemical pathway governing the esterification of xanthophylls seems to be crucial in order to implement strategies for increasing the carotenoid content of crops. In this way, we have proposed tritordeum grains as an excellent plant model to deciphering the functions and significance of the esterification of xanthophyll process in plants, including the characterization of the xanthophylls acyltransferase enzymes (XAT) and the acyl donor molecules (acyl lipids and/or free fatty acids) involved in this still unknown pathway (Mellado-Ortega and Hornero-Méndez, 2012).

As far as we know, to date only one of the parental cereal species of the amphiploid tritordeum, durum wheat, has been fully characterized in relation to the individual carotenoid composition (Abdel-Aal et al., 2007; Atienza et al., 2007b; Blanco et al., 2011; Hentschel et al., 2002). Therefore, the present study was aimed to characterize the carotenoid profile in grains of *H. chilense*, with the aim of expanding

the knowledge about the carotenogenic process in tritordeum, as well as to provide biochemical evidences to support the hypothesis that the origin of esterification pattern of tritordeum is a character mostly derived from the genetic background of this parental (*H. chilense*).

## 2. Materials and methods

### 2.1. Plant material

Grains of *H. chilense* (ascension PI 531781 D-2739) were obtained from the National Small Grains Collection (NSGC) of the National Plant Germplasm System (NPGS) of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS). For comparative purposes grains of a commercial variety of durum wheat (*T. turgidum*, Don Pedro cultivar) and an advanced line of tritordeum (HT621) were also analyzed. HT621 was developed within the Cereal Breeding Program of the Institute for Sustainable Agriculture (IAS-CSIC, Córdoba, Spain) and is deposited at the USDA National Plant Germplasm System (ref. PI 636334), being characterized for presenting a high carotenoid content.

### 2.2. Chemicals and reagents

HPLC-grade methanol, methyl tert-butyl ether (MTBE) and acetone were supplied by BDH Prolabo (VWR International Eurolab, S.L., Barcelona, Spain). HPLC-grade deionized water was produced with a Milli-Q 50 system (Millipore Iberica S.A., Madrid, Spain). The rest of reagents were all of analytical grade.

### 2.3. Extraction of carotenoids

Carotenoid pigments were extracted from tritordeum and durum wheat grains according to Mellado-Ortega and Hornero-Méndez (2012). In the case of *H. chilense*, due to the limiting available material, some modifications were introduced in order to down-scale the procedure. Briefly, the plant material (0.15 g; ca. 50 seeds) was ground with a ball mill (MM400 Retsch) by placing the seeds in a 2 mL safe-lock Eppendorf® tube together with two stainless-steel balls (5 mm diameter) during one minute at 25 Hz rate. Carotenoids were subsequently extracted with 1 mL of acetone (containing 0.1% BHT), centrifuged at 13,500×*g* for 5 min at 4 ºC, and the supernatant was directly used for the chromatographic analysis. Only a one-step solvent treatment was necessary for the complete extraction of pigments (data not shown). All operations were carried out under dimmed light to prevent isomerization and photodegradation of carotenoids. Analyses were carried out in quadruplicate.

**2.4. Pigment identification**

The procedure for the identification of carotenoid pigments and their esters in *H. chilense* was the same as already described in previous works for durum wheat and tritordeum (Atienza et al., 2007b; Mellado-Ortega and Hornero-Méndez, 2012). However, due to the limitation of the available plant material, the identification of carotenoid pigments was mainly based on the chromatographic (retention time) and spectroscopic (UV-visible and MS) properties obtained by HPLC-DAD and HPLC-DAD-MS(APCI+), as well as some micro-scale chemical tests for the determination of the presence 5,6-epoxide, hydroxyl and carbonyl groups.

As described by Mellado-Ortega and Hornero-Méndez (2012) the structural assignment of the lutein esters, including the regioisomers, was mainly based on the fragmentation pattern obtained under the liquid chromatography mass spectrometry

(LC-MS (APCI+)) conditions described below. Moreover, the tentative identification of *cis* isomers of lutein was based on the presence and relative intensity (%$A_B/A_{II}$) of the *cis* peak at about 330-340 nm in UV-visible spectrum, a reduction in the fine structure and a small hypsochromic shift in λmax with respect to the all-*trans* lutein, and the chromatographic behavior in the C18 HPLC column (the *cis* isomers are slightly more retained than the all-*trans* isomer).

Authentic pigment standards of carotenoids were isolated, and purified by means of TLC, from natural sources: β-carotene (β,β-carotene) and zeaxanthin (β,β-carotene-3,3´-diol) were obtained from red pepper (*Capsicum annuum* L.), and lutein (β,ε-carotene-3,3´-diol) from mint leaves (*Mentha arvensis*) (Mínguez-Mosquera and Hornero-Méndez, 1993). Standards of lutein esters isolated and characterized in our previous conducted with tritordeum (Mellado-Ortega and Hornero-Méndez, 2012) were used to confirm the correct structural assignment of the xanthophyll esters in *H. chilense*.

**2.5. HPLC analysis of carotenoids**

HPLC analysis of carotenoids was carried out according to the method of Mellado-Ortega and Hornero-Méndez (2012). The HPLC system consisted of a Waters 2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector, and controlled with Empower2 software (Waters Cromatografía, S.A., Barcelona, Spain). A C18 reversed-phase analytical column (Mediterranea SEA18, 3 μm, 20×0.46 cm; Teknokroma, Barcelona, Spain) was used. Separation was achieved by a binary-gradient elution using an initial composition of 75% acetone and 25% deionised water, which was increased linearly to 95% acetone in 10 min, then raised to 100% in 2 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. An injection

186 volume of 10 μL and a flow rate of 1 mL/min were used. Detection was performed at
187 450 nm, and the online spectra were acquired in the 350-700 nm wavelength range.
188 Quantification was carried out using calibration curves prepared with lutein and β-
189 carotene standards isolated and purified from natural sources (Mínguez-Mosquera &
190 Hornero-Méndez, 1993). Quantification was performed by using calibration curves
191 (peak area at 450nm versus the pigment concentration; range of 0.5-45 μg/ml) prepared
192 with lutein, β-carotene and zeaxanthin standards isolated and purified from natural
193 sources (Mínguez-Mosquera and Hornero-Méndez, 1993). The concentrations of lutein
194 esters and *cis*-isomers of lutein were estimated by using the calibration curve for free
195 lutein. Analyses were carried out in quadruplicate.
196
197 **2.6. Liquid Chromatography-Mass Spectrometry (LC-MS (APCI+))**
198 LC-MS was performed using a chromatographic system consisted of a Waters
199 2690 Alliance chromatograph equipped with a Waters 996 photodiode array detector
200 and coupled to a Micromass ZMD4000 mass spectrometer equipped with a single
201 quadrupole analyzer (Micromass Ltd, Manchester, United Kingdom) equipped with an
202 APCI probe (Atmospheric Pressure Chemical Ionisation). The system was controlled
203 with MassLynx 3.2 software (Micromass Ltd, Manchester, United Kingdom). The
204 chromatographic analysis was carried out according to the method described by
205 Mellado-Ortega and Hornero-Méndez (2012). A C30 reversed-phase column (5 μm,
206 25x0.46 cm; YMC Europe GMBH, Dinslaken, Germany) was used. Separation was
207 achieved by a ternary-gradient elution using an initial composition of 81% methanol
208 (A), 15% MTBE (B) and 4% deionized water (C). The initial composition was linearly
209 changed to 45% A, 53% B and 2% C in 45 min, and subsequently to 9% A and 91% B
210 in 15 min. Initial conditions were reached in 5 min. An injection volume of 20 μL and a

flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra were acquired in the 250-700 nm wavelength range. The mass spectrometer condition parameters were: positive ion mode (APCI+); source temperature, 150 ºC; probe temperature, 400 ºC; corona voltage, 3.7kV; high voltage lens, 0.5kV; and cone voltage, 30V. Nitrogen was used as the desolvation and cone gas at 300 and 50 L/h, respectively. Mass spectra were acquired within the *m/z* 300-1200 scan range.

### 2.7. Statistical analysis.

Basic statistics, mean and standard deviation (SD), were calculated for the results with the Statistica 6.0 software (Statsoft, 2001).

## 3. Results and discussion

**Figure 1** shows the chromatograms corresponding to the pigment extract obtained from *H. chilense* (ascension PI 531781 D-2739), tritordeum (advanced line HT621) and durum wheat (Don Pedro cultivar) grains. The chromatographic and spectroscopic (UV-visible and MS) characteristics of the major carotenoids identified in *H. chilense* are shown in **Table 1,** and **Figure 2** illustrates their corresponding structures. From the chromatograms it was very remarkable the qualitative similarity of the carotenoid profiles of *H. chilense* and tritordeum, with the characteristic presence of lutein esters (mono- and diesters), which it was more noticeable in *H. chilense*. The chromatographic and spectroscopic (UV-visible and MS) properties of the carotenoids and xanthophyll esters detected in *H. chilense* were in total agreement with the identity of those already identified in tritordeum. In this sense, the xanthophyll ester fraction was exclusively composed by lutein esters, corresponding to mono- and diester with

palmitic and/or linoleic acids, namely lutein-3-*O*-linoleate ($[M+H]^+$ at *m/z*=831), lutein-3-*O*-palmitate (*m/z*=808), lutein-3'-*O*-linoleate (*m/z*=831), lutein-3'-*O*-palmitate (*m/z*=808), lutein dipalmitate (*m/z*=1045), lutein dilinoleate (*m/z*=1093), lutein-3'-*O*-linoleate-3-*O*-palmitate (*m/z*=1069) and lutein-3'-*O*-palmitate-3-*O*-linoleate (*m/z*=1069) (Mellado-Ortega and Hornero-Méndez, 2012). As it has been observed in most cereals (Abdel-Aal et al., 2007; Atienza et al., 2007b; Lepage and Sims, 1968; Panfili et al., 2004), including tritordeum and durum wheat, free all-*trans*-lutein (non-esterified) was found to be the major carotenoid (33.8%) in *H. chilense*, being also accompanied by lesser amounts of 9- and 13-*cis* lutein isomers (HPLC peaks 3 and 4). It should be noted that under the assayed chromatographic conditions it was not possible to discriminate between the two possible geometrical isomers at each conjugated double bond of the central carbon chain of lutein, due to its asymmetrical structure, and for that reason the corresponding peaks were tentatively assigned as 9- or 9'- and 13- or 13'-*cis*-lutein, respectively (see Figure 2). In contrast to tritordeum, it is outstanding the presence of zeaxanthin (12.1%) in *H. chilense*, constituting this a common trait with durum wheat, the other parent of the amphiploid tritordeum, being in agreement with previous studies on durum wheat pigments (Atienza et al., 2007b; Hentschel et al., 2002). This aspect is interesting, since although the two cereal parents appear to have the ability to synthesize zeaxanthin, this character is not observed in the amphiploid tritordeum (Mellado-Ortega and Hornero-Méndez, 2012), which could be due to the over-activation of the β,ε-branch of the biosynthetic pathway, which leads to the formation of lutein, at the expense of β,β- branch, leading to the formation of zeaxanthin (Cazzonelli and Pogson, 2010). This possibility seems more likely that the loss of ability to synthesize zeaxanthin by the amphiploid, since tritordeum have detectable levels of β-carotene, its precursor (Mellado-Ortega and Hornero-Méndez, 2012). The reduced levels of β-

carotene and the absence of α-carotene in *H. chilense* suggest that the hydroxylation steps giving way to the formation of lutein and zeaxanthin are very active.

**Table 2** summarizes the quantitative composition of *H. chilense* grains in comparison to durum wheat (Don Pedro cultivar) and tritordeum (HT630 line). The total carotenoid content (6.14 ± 0.12 μg/g) was of the same order to the concentration observed in the HT621 tritordeum line (7.79 ± 0.07 μg/g), and consistent with previous results (Atienza et al., 2007b; Mellado-Ortega and Hornero-Méndez, 2012). Regarding durum wheat, the total carotenoid content was significantly lower (0.87 ± 0.11 μg/g) than for *H. chilense* and tritordeum grains, and in agreement with other studies carried out in durum wheat (Abdel-Aal et al., 2007; Atienza et al., 2007b; Hentschel et al., 2002). This data partially contrasts with some previous works (Álvarez et al., 1999) in which it was determined that *H. chilense* grains presented a markedly higher pigments content than tritordeums, the last ones being located in an intermediate position, with exceptions, between their parents. Total lutein content (free and esterified) accounted for the 87.65% of the total carotenoids, presenting a significant proportion of esters (55.05%), out of which 25.95% and 29.10% were monoesters and diesters, respectively (**Figure 3**). Although the occurrence of lutein esters has been previously reported in wheat, their appearance is very much related to the storage conditions such temperature, relative humidity and time (Ahmad et al., 2013; Farrington et al., 1981; Kaneko et al., 1995; Lepage and Sims, 1968; Mellado-Ortega and Hornero-Méndez, unpublished results). To our knowledge, only tritordeum grains have shown lutein esters in mature seed at harvest (Rodríguez-Suarez et al., 2014), aspect that is currently under investigation in *H. chilense*.

Regioisomers of lutein monoesters at the position 3 of the β-end ring (lutein-3-*O*-linoleate and lutein-3-*O*-palmitate) were found in higher concentration than the

corresponding monoesters at the position 3' of the ε-end ring (lutein-3'-*O*-linoleate and lutein-3'-*O*-palmitate), which is consistent with the lutein monoester regioisomers profile described in advanced tritordeum lines (Mellado-Ortega and Hornero-Méndez, 2012). In the case of the diesters fraction, a major contribution of the homodiester with palmitic acid (lutein dipalmitate) was observed compared to corresponding with linoleic acid (lutein dilinoleate), suggesting a greater affinity for the esterification with palmitic acid. The results, obtained in *H. chilense*, corroborate the preferential acylating action over the β-end ring of lutein compared to the ε-end ring, as well as the selectivity for palmitic acid of the enzyme systems (XAT: Xanthophyll acyltranferase) which are involved in esterification reaction (Mellado-Ortega and Hornero-Méndez, 2012). It is important to note that the chromatographic peak assigned as the lutein linoleate-palmitate heterodiester consisted of two regioisomers, lutein-3'-*O*-linoleate-3-*O*-palmitate and lutein-3'-*O*-palmitate-3-*O*-linoleate, and hence its greater content compared to the other two homodiesters. The chromatographic conditions did not allow the chromatographic resolution of the two regioisomers, and therefore we cannot establish whether there are differences in relative abundance between the two.

## 4. Conclusions

*H. chilense* has been consistently considered as the parent mostly responsible of the high carotenoid content of tritordeum (Álvarez et al., 1998; Atienza et al., 2004, 2007a; Rodríguez-Suarez and Atienza, 2012). The carotenoid profile, particularly the lutein esters, presented in this study confirm this affirmation, being the first time that the individual carotenoid profile of this cereal has been characterized. Additionally, our data supports the hypothesis of the origin of esterification of lutein in tritordeum as a character mostly derived from the *H. chilense* genetic background. The use of wild

311 relatives of common cereals as sources of genetic variability and contribution of
312 favorable agronomic traits for crops is to be one of the most important strategies in plant
313 improvement through breeding. In this sense *H. chilense* represents a valuable source of
314 genes for increasing carotenoid content in wheat, being tritordeum the vector used for
315 the transfer of the associated characters (Rodríguez-Suarez et al., 2010). The detailed
316 characterization of the carotenoid composition of *H. chilense*, reported in this work,
317 may be useful to optimize its use in the field of biofortification of cereals through the
318 increase of the carotenoid content.
319

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## Figure captions

**Figure 1.** C18 reversed-phase HPLC chromatogram obtained for a carotenoid extract prepared from *H. chilense* (PI 531781) (**A**), tritordeum (HT621) (**B**), and durum wheat (Don Pedro) (**C**) grains. Peak identities: **1**. all-*trans*-zeaxanthin; **2**. all-*trans*-lutein; **3.** 9-*cis*-lutein/9'-*cis*-lutein; **4.** 13-*cis*-lutein/13'-*cis*-lutein; **5**. lutein-3'-*O*-linoleate; **6**. lutein-3-*O*-linoleate; **7**. lutein-3'-*O*-palmitate; **8**. lutein-3-*O*-palmitate; **9**. all-*trans*-β-carotene; **10**. lutein dilinoleate; **11**. lutein-3'-*O*-linoleate-3-*O*-palmitate and lutein-3'-*O*-palmitate-3-*O*-linoleate; **12**. lutein dipalmitate. Detection wavelength was carried out at 450 nm.

**Figure 2.** Chemical structures of the carotenoid pigments identified in *H. chilense* grains (Numbers according to Figures 1).

**Figure 3.** Distribution of free and esterified lutein fractions (monoesters and diesters) in grains of *H. chilense* (PI 531781). Data are the mean (n=4).

**Highlights**

- Carotenoid profile of *Hordeum chilense* grains has been analysed for the first time.
- Lutein is the major carotenoid pigment in *H. chilense* grains.
- The esterification pattern of lutein in *H. chilense* and tritordeum is identical.
- *H. chilense* genetic background provides the higher carotenoid content to tritordeum.

**Table 1**

**Table 1.** Chromatographic (HPLC), spectroscopic properties (UV-visible and MS) and micro-scale chemical tests for specific functional groups for the carotenoid pigments presents in *Hordeum chilense* grains.

| | | | UV-visible spectrum[b] | | | HPLC/APCI(+) MS | Micro-scale chemical tests for functional groups | | |
|---|---|---|---|---|---|---|---|---|---|
| Peak[a] | Carotenoid | Retention time (min) | $\lambda_{max}$ (nm) in HPLC mobile phase | III/II (%) | $A_B$/II (%) | fragmentation pattern *m/z* (fragment; relative abundance (%)) | 5,6-Epoxide | Hydroxyl | Carbonyl |
| 1 | all-*trans*-Zeaxanthin | 9.32 | (428), 454, 482 | 8 | 0 | 569.6 ($[M+H]^+$; 100), 551 ($[M+H-18]^+$; 17), 533 ($[M+H-18-18]^+$; 5) | - | + | - |
| 2 | all-*trans*-Lutein | 9.52 | 428, 448, 476 | 65 | 0 | 569.6 ($[M+H]^+$; 6), 551 ($[M+H-18]^+$; 100), 533 ($[M+H-18-18]^+$; 5) | - | + | - |
| 3 | 9-*cis*-Lutein or 9'-*cis*-Lutein | 10.15 | 330, 420, 442, 471 | 67 | 21 | 569.6 ($[M+H]^+$; 5), 551 ($[M+H-18]^+$; 100), 533 ($[M+H-18-18]^+$; 5) | - | + | - |
| 4 | 13-*cis*-Lutein or 13'-*cis*-Lutein | 10.35 | 330, 418, 441, 469 | 45 | 44 | 569.6 ($[M+H]^+$; 5), 551 ($[M+H-18]^+$; 100), 533 ($[M+H-18-18]^+$; 5) | - | + | - |
| 5 | Lutein-3'-*O*-linoleate | 15.32 | 424, 447, 476 | 65 | - | 831.3 ($[M+H]^+$; 30), 551.3 ($[M+H-280]^+$;100) | - | + | - |
| 6 | Lutein-3-*O*-linoleate | 15.44 | 424, 447, 476 | 64 | - | 831.3 ($[M+H]^+$; 12), 813.8 ($[M+H-18]^+$; 100), 533.3 ($[M+H-18-280]^+$; 47) | - | + | - |
| 7 | Lutein-3'-*O*-palmitate | 15.92 | 424, 447, 476 | 65 | - | 807.6 ($[M+H]^+$; 25), 551.4 ($[M+H-256]^+$; 100) | - | + | - |
| 8 | Lutein-3-*O*-palmitate | 16.10 | 424, 447, 476 | 65 | - | 807.6 ($[M+H]^+$; 9), 789.7 ($[M+H-18]^+$; 100), 533.3 ($[M+H-18-256]^+$; 44) | - | + | - |
| 9 | all-*trans*-β-Carotene | 16.37 | (428), 452, 480 | 10 | 0 | 537.4 ($[M+H]^+$; 100), 445.6 ($[M+H-92]^+$; 21) | - | - | - |
| 10 | Lutein dilinoleate | 19.17 | 424, 447, 476 | 65 | - | 1092.9 ($[M+H]^+$; 19), 813.6 ([M+H- | - | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 280]$^+$; 100), 533.4 ([M+H-280-280]$^+$; 62) | | | |
| 11 | Lutein-3'-*O*-linoleate-3-*O*-palmitate<br>and<br>Lutein-3'-*O*-palmitate-3-*O*-linoleate | 20.52 | 424, 447, 476 | 64 | - | 1069.6 ([M+H]$^+$; 8), 813.3 ([M+H-256]$^+$; 24), 789.7 ([M+H-280]$^+$; 100), 533.3 (M+H-256-280]$^+$; 60)<br>1069.6 ([M+H]$^+$; 4), 813.3 ([M+H-256]$^+$; 100), 789.6 ([M+H-280]$^+$; 16), 533.3 ([M+H-280-256]$^+$; 60) | - | - | - |
| 12 | Lutein dipalmitate | 22.18 | 424, 447, 476 | 64 | - | 1045.7 ([M+H]$^+$; 12), 789.7 ([M+H-256]$^+$; 100), 533.4 ([M+H-256-256]$^+$; 65) | - | - | - |

a. Peak numbers are according to Figures 1 and 2.
b. III/II (%): Spectral fine structure, defined as the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100. $A_B$/II (%): Intensity of the *cis*-peak, defined as the ratio of the height of the *cis*-peak ($A_B$) and that of the middle main absorption peak (II) multiplied by 100.

**Table 2.** Carotenoid composition (μg/g fresh weight) in grains *Hordeum chilense* (ascensions PI531781) *Triticum turgidum* cv. *durum* (Don Pedro cultivar) and *tritordeum* (HT621).

| HPLC Peak [a] | Pigment | Concentration (μg/g fresh weight)[b] | | |
|---|---|---|---|---|
| | | ***H. chilense* (ascensions PI531781)** | **Durum wheat (Don Pedro cultivar)** | **Tritordeum (advanced line HT621)** |
| 1 | all-*trans*-Zeaxanthin | 0.74 ± 0.01 | 0.10 ± 0.01 | - |
| 2 | all-*trans*-Lutein | 2.07 ± 0.04 | 0.62 ± 0.09 | 4.18 ± 0.09 |
| 3 | 9-*cis*-Lutein or 9´-cis-Lutein | 0.17 ± 0.00 | 0.05 ± 0.00 | 0.19 ± 0.00 |
| 4 | 13-*cis*-Lutein or 13´-cis-Lutein | 0.18 ± 0.01 | 0.07 ± 0.00 | 0.30 ± 0.01 |
| 5+6 | Lutein monolinoleate | 0.72 ± 0.01 | - | 0.82 ± 0.00 |
| 5 | Lutein-3'-O-linoleate | 0.23 ± 0.01 | - | 0.14 ± 0.00 |
| 6 | Lutein-3-O-linoleate | 0.49 ± 0.01 | - | 0.68 ± 0.00 |
| 7+8 | Lutein monopalmitate | 0.68 ± 0.01 | - | 1.36 ± 0.01 |
| 7 | Lutein-3'-O-palmitate | 0.30 ± 0.01 | - | 0.43 ± 0.00 |
| 8 | Lutein-3-O-palmitate | 0.38 ± 0.01 | - | 0.93 ± 0.01 |
| 9 | all-*trans*-β-Carotene | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.07 ± 0.00 |
| 10 | Lutein dilinoleate | 0.31 ± 0.02 | - | 0.12 ± 0.00 |
| 11 | Lutein-3'-*O*-linoleate-3-*O*-palmitate and Lutein-3'-*O*-palmitate-3-*O*-linoleate | 0.75 ± 0.02 | - | 0.39 ± 0.01 |
| 12 | Lutein dipalmitate | 0.51 ± 0.01 | - | 0.37 ± 0.00 |
| | Lutein monoesters | 1.40 ± 0.01 | - | 2.18 ± 0.01 |
| | Lutein diesters | 1.57 ± 0.05 | - | 0.88 ± 0.01 |
| | Total lutein | 5.38 ± 0.11 | 0.74 ± 0.10 | 7.73 ± 0.07 |
| | Total lutein esters | 2.96 ± 0.06 | - | 3.06 ± 0.01 |
| | Total carotenoids | 6.14 ± 0.12 | 0.87 ± 0.11 | 7.80 ± 0.07 |

[a] Peak numbers are according to Figures 1 and 2.

[b] Data are the mean ± standard deviation (n=4)

**Figure 1**

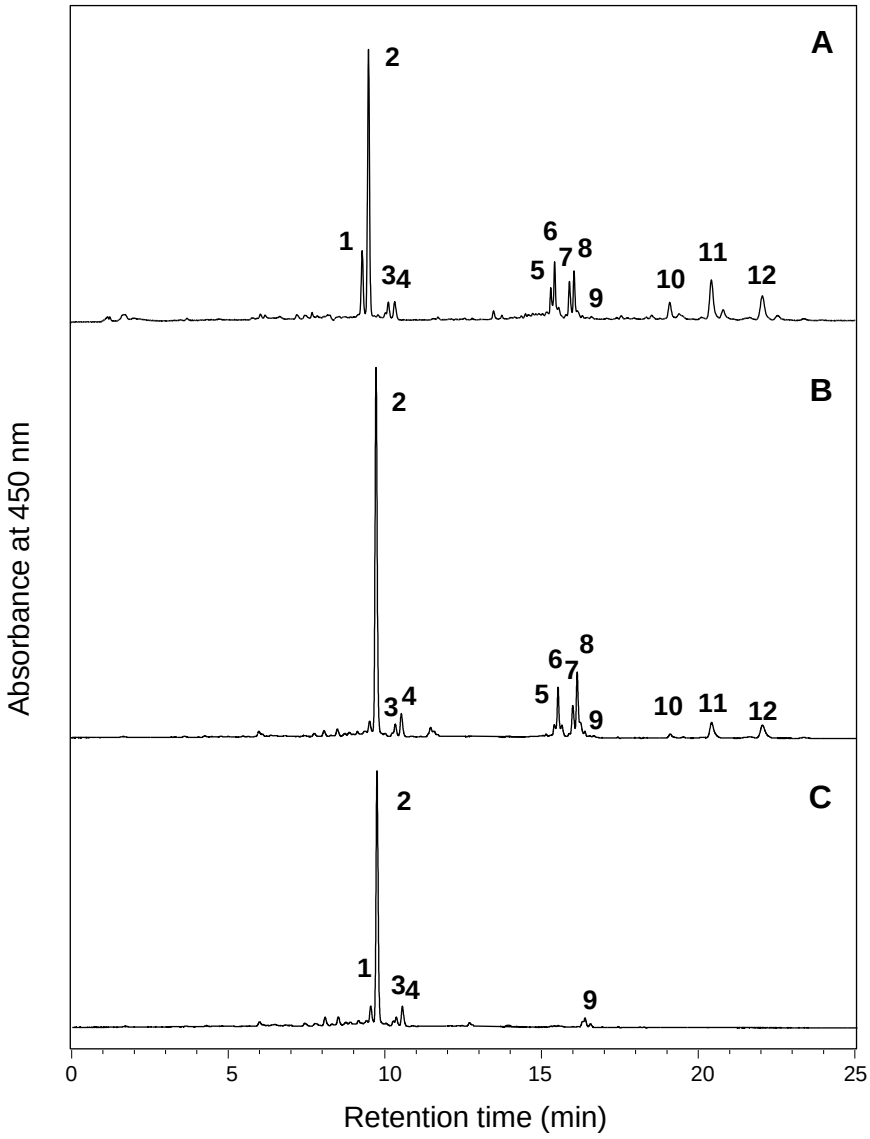
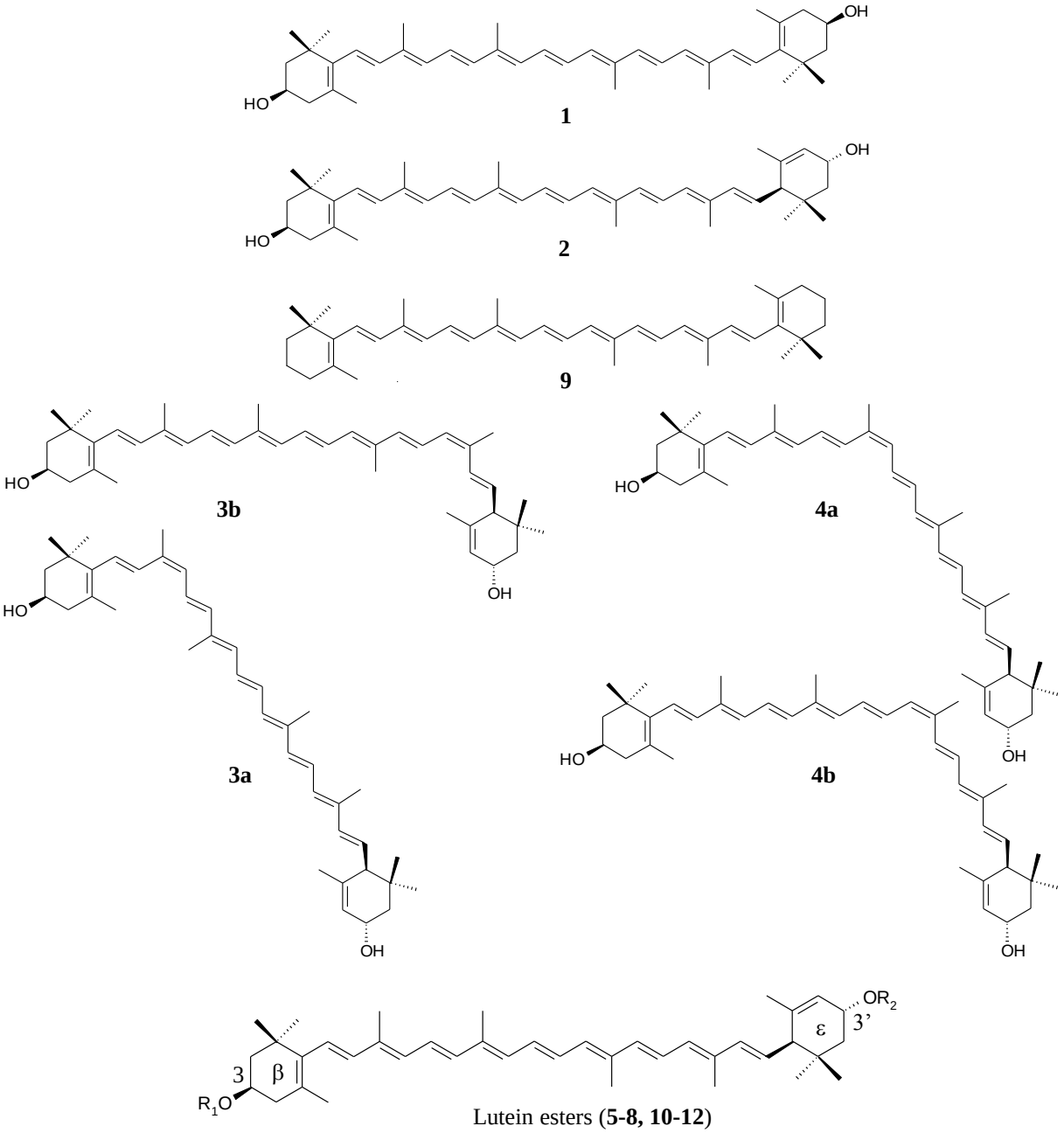

A
B
C
1
2
3
4
5
6
7
8
9
10
11
12
Absorbance at 450 nm
0
5
10
15
20
25
Retention time (min)

**Figure 2**




Lutein esters (**5-8, 10-12**)

| $R_1$ (at carbon 3, β- ring) | $R_2$ (at carbon 3', ε- ring) | Lutein esters |
|---|---|---|
| H | Linoleoyl (C18:2) | Lutein-3'-*O*-linoleate (**5**) |
| Linoleoyl (C18:2) | H | Lutein-3-*O*-linoleate (**6**) |
| H | Palmitoyl (C16:0) | Lutein-3'-*O*-palmitate (**7**) |
| Palmitoyl (C16:0) | H | Lutein-3-*O*-palmitate (**8**) |
| Linoleoyl (C18:2) | Linoleoyl (C18:2) | Lutein dilinoleate (**10**) |
| Palmitoyl (C16:0) | Linoleoyl (C18:2) | Lutein-3'-*O*-linoleate-3-*O*-palmitate (**11**) |
| Linoleoyl (C18:2) | Palmitoyl (C16:0) | Lutein-3'-*O*-palmitate-3-*O*-linoleate (**11**) |
| Palmitoyl (C16:0) | Palmitoyl (C16:0) | Lutein dipalmitate (**12**) |

**Figure 3**

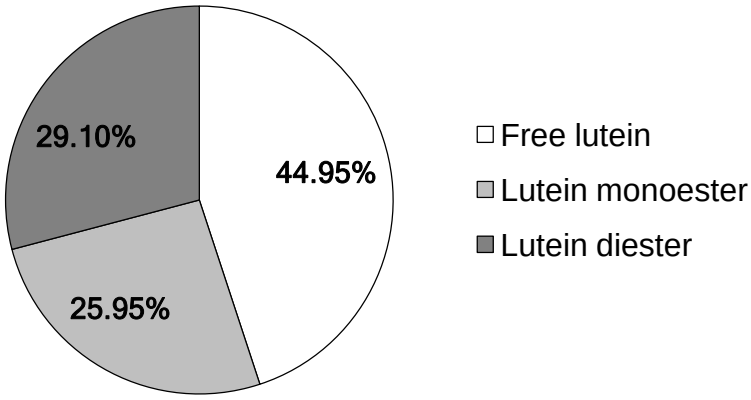

29.10%
44.95%
25.95%
Free lutein
Lutein monoester
Lutein diester

**Figure 1 color for Web version**

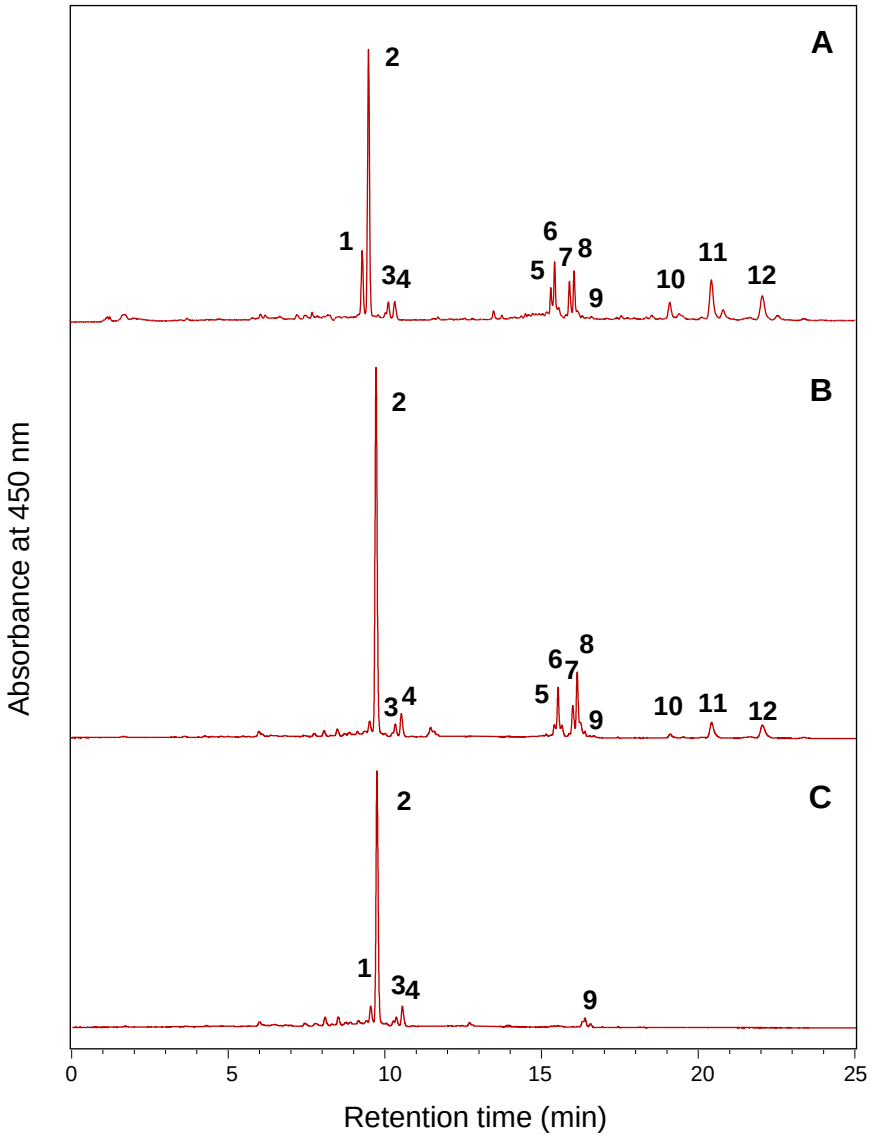


**Figure 3 color for Web version**